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SMC1 inhibition results in FRA3B expression but has no effect on its delayed replication

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Abstract

Cellular processes involved in fragile site expression have been investigated by studying the effect on the replication pattern of the commonest fragile site FRA3B of RNA interference (RNAi)-mediated sister maintenance chromosome 1 (*SMC1*) inhibition in normal human fibroblasts. Replication timing of FRA3B in G_2 was studied by bromodeoxyuridine (BrdU) labeling for the final 2 h of cell culture whereas in the S phase was investigated by a fluorescence in situ hybridization (FISH)-based approach through the analysis of clones spanning the FRA3B region. Results showed that FRA3B is normally late replicated even though it is not expressed in untreated cells. On the other hand, *SMC1* inhibition leads to FRA3B expression even if the percent of late replicated cells is comparable to control cells. These results obtained by analysing the commonest fragile site suggest that *SMC1* plays a role in protecting late replicating regions from stresses occurring in the final steps of genome replication and that delayed replication is necessary but not sufficient for inducing fragile site expression.

Keywords: Fragile sites; FRA3B; *SMC1*; Delayed replication

1. Introduction

Fragile sites are breakage-prone regions on human chromosomes, which result when cells are exposed to specific chemical agents or tissue culture condition. They are divided into two classes, rare and common, on the basis of their relative occurrence in the population. Common fragile sites have drawn considerable attention for their involvement in tumorigenesis. This notion is supported by the observations that some of them map to cancer breakpoints [1,2] and are general targets of many mutagens and carcinogens [3,4]. The majority of common fragile sites are induced by aphidicolin, an inhibitor of DNA polymerase α, ε and δ [5–7] and their frequency increase after caffeine or campthotecin treatment, the latter being able to also induce new fragile sites [8]. Five common fragile sites, namely, FRA3B, FRA6E, FRA7G, FRA7H and FRA16D, have been cloned and characterized and they span from hundreds of kilobases to 4 Mb [9,10].

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Among fragile sites, FRA3B is the commonest in humans. FRA3B is spanned by the fragile histidine triad (FHIT) gene, which codes for a diadenosine polyphosphate hydrolase (reviewed in [9]). Much evidence supports the association of FRA3B with tumorigenesis. FRA3B is a target of many mutagens and carcinogens known to act via different molecular mechanisms, including nucleotide alkylating agents, DNA replication or transcription inhibitors and hypomethylating agents [11]. Furthermore, subjects professionally exposed to pesticides have increased fragility at FRA3B suggesting that its expression is influenced by environmental factors [3]. FRA3B is, also, involved in chromosomal aberrations undistinguishable from those occurring in tumor cells. FHIT is expressed in most normal tissue whereas its expression is altered in tumors such as lung, colon, breast and ovary (reviewed in [9]). Inactivation of FHIT is one of the most frequent alterations found in tumors. For example, it is the target of a reciprocal translocation t(3;20)(p14;p11) in a breast tumor cell line leading to loss of FHIT expression [12]. It has been suggested that FHIT acts as a tumor suppressor by modulating the apoptotic process [13]. In spite of these extensive studies, the molecular basis for FRA3B expression is still unclear. The understanding of the molecular basis of fragility at FRA3B, therefore, might help us to gain a better insight into tumorigenesis.

Recently, we showed that inhibition of sister maintenance chromosome 1 (*SMC1*) by RNA interference (RNAi) or antisense oligonucleotides, are sufficient to induce chromosomal aberrations in normal human fibroblasts, most of which are located at fragile site chromosome bands. Aphidicolin (APH) plus *SMC1* inhibition increased aberration frequency, due to the synergistic effect of the two treatments [14,15]. Smc1 is a subunit of cohesin, a protein complex, which consists of Smc1, Smc3 and two non-Smc subunits (Scc1 and Scc3). The Smc1-Smc3 heterodimer has also been found to promote repair of gaps and deletions and the cohesin complex is required for postreplicative double strand breaks (DSBs) repair in *Saccharomyces cerevisiae* [16–19].

Several reports suggest that fragile sites could be late-replicating regions [8,20–23]. To elucidate the mechanisms involved in FRA3B expression, we analysed the replication pattern of the common fragile site FRA3B after *SMC1* inhibition by RNAi. Replication timing of FRA3B was studied by two different approaches. Replication in G_2 phase was visualized by labeling fibroblasts with bromodeoxyuridine (BrdU) for the last 2 h of cell culture whereas replication in the S phase was investigated with a fluorescence in situ hybridization (FISH)-based approach through the analysis of clones spanning the FRA3B region. Our results confirm that FRA3B is normally replicated late and that in untreated cells this is not sufficient for fragile site expression. On the other hand, *SMC1* inhibition induces FRA3B expression even in the absence of a delayed replication. Since *SMC1* has been implicated in G_2/M checkpoint in an *Ataxia telangiectasia* and Rad3-related (ATR)-dependent manner, our data suggests that *SMC1* could play a pivotal role in the last events of normal replication, such as termination of DNA replication. In this regard, either a prolonged stress, resulting from APH exposure, or an *SMC1* function defect could lead to fragile site expression due to checkpoint failure.

2. Material and methods

2.1. Cell culture

Normal primary human fibroblasts were grown in Dulbecco's minimal essential medium (DMEM, Gibco BRL) supplemented with 10% fetal calf serum and antibiotics in a humidified 5% CO_2 atmosphere.

2.2. RNAi synthesis, cell treatment and fragile site expression

RNAi corresponding to *SMC1* mRNAs was designed as recommended [24] with two base overhangs. The following gene-specific sequence was used: RNAi-*SMC1*, 5′-AUC UCA UGG AUG CCA UCA G dTT-3′. Scrambled RNA was constructed as control. Cells (at 40–60% confluence) were transfected with RNAis by using siPort Amine (Ambion) or treated with APH (0.4 μM) for 26 h, alone or in combination with *SMC1* inhibitor.

2.3. Cytogenetic analysis

Exponentially growing fibroblasts were treated with colcemid (0.05 μg/ml, Gibco BRL), harvested, incubated with KCl 0.075 M and fixed in methanol:acetic acid (3:1). Chromosome preparations were G-banded according to the trypsin standard digestion procedure.

2.4. Visualization of late replication in FRA3B

The visualization of late replication in the FRA3B region was performed according to a published protocol with minor modifications [20]. Cells were labeled with BrdU at a final concentration of 10 μM for the last 2 h of cell culture. Metaphase spreads were denaturated by incubation for 2 min in a mixture of ethanol/0.1 M NaOH (2:5). The denaturated slides were then permeabilized with 5% TritonX100 in PBS for 5 min followed by incubation with 3% bovine serum albumin (BSA) for 30 min. The slides were incubated with anti-BrdU antibody in 1% BSA in PBS. After three washings with PBS, slides were incubated with fluorescein-labeled anti-mouse IgG for 1 h. The slides were then washed three times with PBS and counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) allowing the identification of chromosomes.

2.5. Probes

30G4 and 94D19 are RP-11 BAC clones (Research Genetics) that map to the FRA3B "active region" [10]. Q2A2, D13C2 and Q71A3, cosmid clones from the acute myeloid leukemia 1 (AML1) gene, as control region, were obtained from the Institute for Molecular Biotechnology (Jena, Germany).

2.6. Fluorescence in situ hybridization

Probes were labeled by nick translation using directly labeled nucleotide (Vysis) or biotin-16-dUTP or digoxigenin-11-dUTP (Roche). Biotin- and digoxigenin-labeled probes were detected by FITC-conjugated avidin and rhodamine-conjugated antibodies, respectively. Cells in S phase were identified by BrdU incorporation (Becton Dickson). Nuclei were counterstained with DAPI. The slides were examined using an epifluorescence microscope (DMRXA, Leica). 300 nuclei were scored for the presence of two singlet signals (SS), one single and one double signal (SD), or two signal doublets (DD).

3. Results and discussion

Recently, we showed that *SMC1* inhibition by RNAi leads to fragile site expression [14]. Indeed, on the most frequently expressed fragile site FRA3B occurred as 15.4% (4/26) of the aberrations and this frequency increased to 21.2% (38/179) after APH plus RNAi-*SMC1* combined treatment [14]. Here, to elucidate the mechanisms involved in FRA3B expression, we studied, using two different approaches, its replication status after *SMC1* inhibition. First, normal human fibroblasts were labeled with BrdU for the last 2 h of cell culture and indirect immunofluorescence was used for detecting BrdU in metaphases. Since BrdU is incorporated into DNA 2 h before preparing metaphases, the BrdU-positive regions would represent late replicating regions of the genome. Untreated cells showed 8% late replication signals and a comparable rate (10%) was found after *SMC1*-RNAi inhibition. On the contrary, after APH treatment alone this percentage increased to 20% ($P = 0.02$, χ^2-test) while a further increase (35%) was seen with APH plus RNAi-*SMC1* combined treatment ($P < 10^{-3}$; Fig. 1A and Table 1).

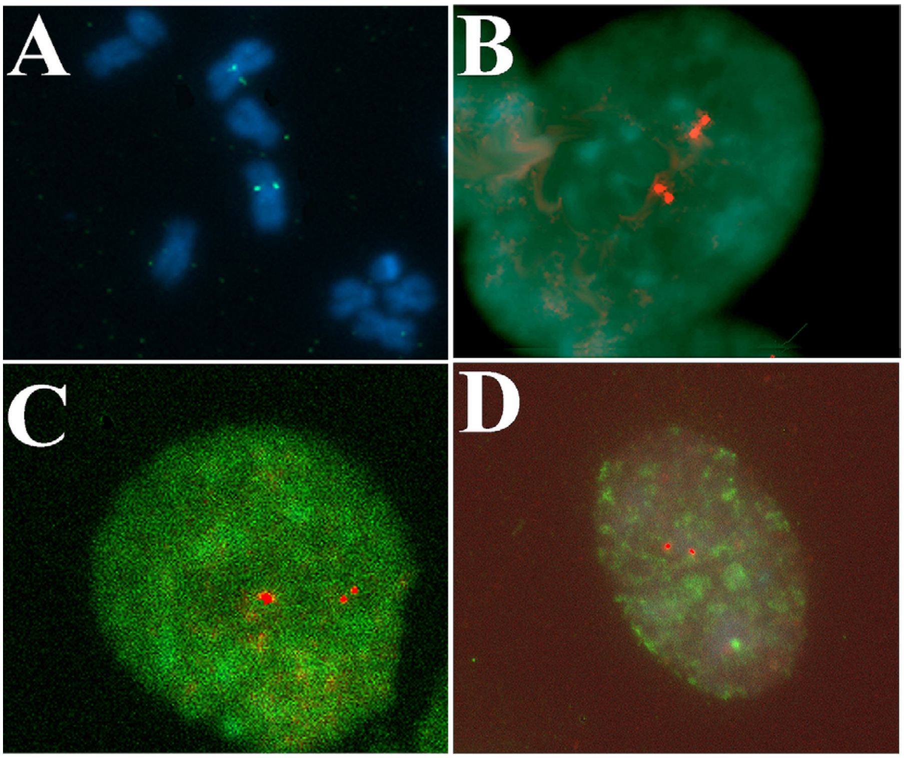

Fig. 1. Late replication in the FRA3B region. (A) BrdU labeled DNA detected by an immunofluorescent procedure representing late replication region, (B) S phase nucleus in which FRA3B has completed replication revealing two sets of signal doublets, (C) immunostaining showing asynchronous replication with a single and a doublet signal and (D) immunostaining showing two unreplicated FRA3B alleles with SS signals.

Table 1
Late replication on chromosome 3 visualized as a BrdU-incorporating region

	Signals	*P*
Control	8	
Control scrambled RNAi-*SMC1*	7	n.s
APH	20	0.02
RNAi-*SMC1*	10	n.s
APH plus RNAi-*SMC1*	35	$<10^{-3}$

n.s. = not significant.

In addition to G_2 replication, we studied the FRA3B replication timing status in the S phase with a different FISH-based approach through the analysis of clones spanning the FRA3B region. In untreated cells, depending on the probe used for the analysis, the percentage of nuclei showing DD ranged from 31 to 38%, while the percentage of cells with SD signals ranged from 22 to 31% and that of SS signals was between 38 and 40%. Similar data was found also after *SMC1* inhibition. In fact, the percentage ranged from 32 to 40 for DD, from 22 to 27 for SD and from 38 to 41 for SS signals. As expected, treatment with APH induced an increase in cells showing both SS and SD signals. In fact, they ranged from 41 to 46% for SS and from 26 to 29% for SD. Combined treatment (APH plus RNAi-*SMC1*) led to a further increase, with the percentage of cells showing SS ranging from 50 to 55% and that of SD ranging from 30 to 32% (Fig. 1B–D and Fig. 2A). The analysis of AML1 clones, used as a control region, showed that most alleles were normally replicated and treatment with APH, alone or combined with RNAi-*SMC1*, had no effect (Fig. 2B). Statistical analysis showed a highly significant difference in signal distribution for both APH and APH plus RNAi-*SMC1* between FRA3B and control clones (in both cases, $P < 10^{-3}$).

Here, by detecting BrdU labeled DNA, we show that FRA3B, the commonest fragile site in humans, is normally late replicating, confirming previous data [22] and that *SMC1* inhibition does not lead to an increase in late replication events, even if the treatment induced FRA3B expression. On the contrary, an increase in cells with delayed replication was seen with APH treatment. The combined treatment (APH plus RNAi-*SMC1*) resulted in a further reduction of DD and DS signals. These observations further support the suggestion that conditions for fragile sites expression require a delayed replication leading to incomplete chromosome condensation thus resulting in gaps or breaks for the experimental conditions used.

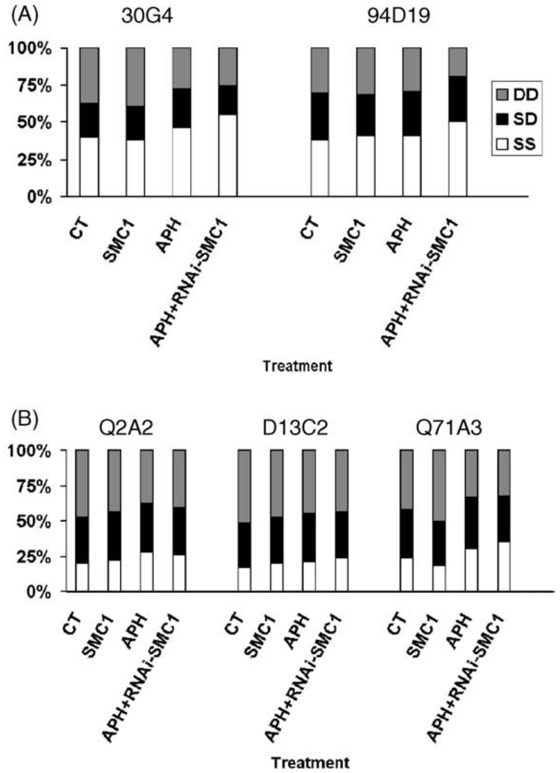

Fig. 2. Asynchronous replication pattern along the FRA3B in S phase cells by in situ hybridization in normal human fibroblasts. (A) Percentage of alleles showing SS, SD and DD signal in untreated, APH treated and APH plus RNAi-*SMC1* treated cells by using 30G4 and 94D19 clones as probes and (B) the same analysis was performed through Q2A2, D13C2 and Q71A3 clones as early replicated control region.

On the other hand, the finding that *SMC1* led to FRA3B expression without delayed replication allows us to gain new insight into fragile site expression.

In addition to chromosome cohesion, *SMC1* has been recently implicated in DNA repair and chromosome dynamics [16,17]. *SMC1* is a component of the DNA damage response that acts as a downstream effector in the ATR checkpoint pathway. In fact, *SMC1* was recently shown to be a target of the ATR kinase acting through phosphorylation of Ser966 occurring in an ATR dependent manner after APH treatment [14]. Experiments performed on an *SMC1* phosphorylation-deficient, from an *Atr* mouse knockout, and with cell lines deriving from Seckel syndrome showed a high frequency of chromosome aberrations. In addition, very low doses of APH are required to induce fragile sites suggesting that both these genes play a role in stabilizing stalled replication forks [25–27]. In our experimental conditions, FRA3B expression, after *SMC1* inhibition, could be linked to physiologic processes requiring residual DNA synthesis.

Although these steps are not completely clear, termination of DNA replication could be involved. Up to now, all characterized common fragile sites cover a large portion of the genome, from 1 to 4 Mb [9,10], and it is presumable that several replicons fall in these regions. The inhibition of *SMC1* could lead to the bypassing of the G_2/M checkpoint, with cells proceeding to mitosis with many un- or under-replicated regions. Alternatively, *SMC1* could play a structural role in maintaining the stability of late replicating regions. In this regard, either a prolonged stress causing a collapse of stalled replication forks, resulting from APH exposure, or an *SMC1* function defect could lead to fragile site expression due to checkpoint failure.

In conclusion, fragile sites could be the consequence of a large genome replication such as the human one. In this regard, *SMC1,* playing a role both in chromosome structure and DNA repair, could protect these late replicating regions from damage due to stalled forks of which fragile sites are the cytogenetic expression.

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